## REMARKS

Claims 1, 5-15, and 18-44 are pending in the present application. Claims 9, 12-13, 22-28, and 31-40 were previously withdrawn from consideration as drawn to a non-elected invention. In the response filed on September 28, 2005, Applicant's representative asked the Examiner to reconsider the withdrawal of claims 39 and 40. With the Office Action mailed on March 21, 2006, Examiner listed claims 39-44 as being under consideration. As such, Applicant thanks the Examiner for his consideration for claims 39 and 40. By virtue of this response, claims 1, 5-8, 10, 11, 14-15, 18-21, 29-30, and 39-44 are under consideration. Amendment and cancellation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented.

### *Rejection under 35 U.S.C. § 112*

Claim 44 stands rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as his invention.

Claim 44 reads: "[t]he adenovirus vector of claim 43 wherein said transgene is substituted for the E3 region in parallel orientation." The Examiner alleges that the term "parallel orientation" is being used by the claim "to mean 'expressed,' while the accepted meaning does not make any sense in this context." Applicant respectfully disagrees with the Examiner's assertions. Claim 44 does not recite the term "expressed" nor does it require that the transgene must be expressed to meet the claim limitations. As such, Applicant does not understand why the Examiner contends that Applicant is using the term "parallel orientation" to mean expressed in this claim.

Support for "parallel orientation" is found in Example 5 at paragraph 123. The specification teaches that "[t]he recombinant BAV303 contained the HE cDNA sequence substituting for BAV-3 E3 in parallel orientation so as to allow expression from endogenous promoters." Thus, claim 44, read in context of the teaching of the specification, means that the transgene is substituting for the E3 region such that the transgene reads in the same direction (i.e.,

parallel) as the E3 region. If the E3 region codes in the 5' to 3' direction, this would mean that the transgene which is being put into the E3 region would also have to be inserted in such an orientation such that its coding also runs in the 5' to 3' direction. Applicant asserts that the term "parallel orientation" is commonly used by those of skill in the art to refer to this type of placement of genes. Example 5 takes this concept one step further by showing that the replacement of E3 region by the transgene HE yielded little to no expression when the expression vector did not contain an intron 5' to the transgene (as is claimed in claim 1).

The Examiner alleges that the specification uses this term to indicate that the transgene is expressed. Applicant disagrees with the Examiner because the sentence explaining the substitution of the HE gene for the BAV-3 E3 states that the substitution of the HE gene was inserted in the parallel orientation "***so as to allow*** expression" (emphasis added). This means that when one of skill in the art is making these expression vectors, he/she has to be careful to get the orientation of the transgene correct *so as to allow* expression. One of skill in the art would easily understand that putting in a transgene backwards (i.e., 3' to 5') could mean that the cloned expression vector would not express the transgene since the original E3 region runs 5' to 3' and the transgene has been put in backwards (3' to 5'). As such, the specification teaches that the transgene should be placed in the same orientation as the E3 gene. Since the E3 gene runs 5' to 3' then the transgene being substituted for the E3 gene should also be placed in such an orientation that is also 5' to 3'. This is what paragraph 123 refers to as "parallel orientation" since both the E3 gene and the transgene are running in parallel with each other. If the transgene is placed into the vector such that it runs in the opposite direction of what E3 does, then it is commonly referred to as being in an "antiparallel orientation."

To show the Examiner that one of skill in the art uses the term "parallel orientation" in the manner described above, Applicant has included the Yarosh et al. reference (*Vaccine*, 14: 1257-1264 (1996)). The Yarosh reference is discussed in greater detail below but for purposes of traversing this indefiniteness rejection, Applicant calls the Examiner's attention to the first paragraph of the "Generation of recombinant viruses" section of the Materials and Methods section (page 1258). Here, the authors describe placing the resultant expression cassette in a "antiparallel

orientation" in the E3 region of a hAd5 virus vector so that "neither the endogenous E3 promoter nor hAd5 MLP can be used to transcribe RG in this construct." The authors use this type of parallel/antiparallel terminology again in the first full paragraph, right hand column of page 1258 to describe the insertion of an Xba 1 fragment into an "antiparallel orientation" relative to the E3 promoter. Yet another example of the use of "parallel orientation" is seen in the Yarosh reference in the first paragraph under the Results section ("polyA addition sequence in the E3 *parallel orientation*" (emphasis added)).

In view of the foregoing, Applicants submit that the term "parallel orientation" is not indefinite because the specification clearly defines the term such that one of skill in the art could understand what was meant by claim 44. As such, Applicants respectfully request that this rejection be withdrawn.

***Claim Rejection under 35 U.S.C. § 103***

Claims 1, 5-8, 10-11, 14-15, 18-21, 29-30, and 39-44 stand rejected for being obvious over Lusky et al (which is in French) in view of Matthews et al. and further in view of Kaufman et al.

***Basic Requirements for Prima Facie Case of Obviousness***

Applicant traverses this rejection on the basis that the Examiner has not shown the three basic elements required for a *prima facie* case of obviousness: (1) a suggestion or motivation to combine the references; (2) there must a reasonable expectation of success; and (3) the prior art references (or references when combined) must teach or suggest all the claim limitations. MPEP § 2143.

1. There is No Motivation to Combine the Lusky, Matthews and Kaufman References Because the References Teach Different Aspects of Adenoviruses That Are Not Related to the Invention as Currently Claimed.

The Examiner alleges that Lusky teaches BAV3 vectors (Figures 15A and 15B, column 12, and Example 4). As Applicant pointed out in the previous response, the Lusky reference (WO 99/61638) is in French. However, U.S. Patent No. 6,479,290 ('290 patent) appears to be filed in the USPTO in English under § 371 based on WO 99/61638. As such, Applicant has used this '290 patent as a substitute for the Lusky reference.

The Examiner states that the Applicant cannot show non-obviousness by attacking the references individually where the rejection is based on a combination of references. In response, Applicant points out that in order for the Applicant to show that there is no motivation to combine the references, each of the references has to be discussed in turn before reaching the conclusion that there is no motivation to combine all the references.

Applicant notes that there are no figures in the '290 patent (nor in original WO 99/61638). As such, Applicants are unclear as to why the Examiner referred to Figures 15A and 15B when there appears to be no figures filed in this application. Furthermore, column 12 of the '290 patent does not appear to discuss the replication-competent bovine adenovirus vector with a transgene inserted into the E3 region that is currently being claimed. Column 12 of the '290 patent discusses, *inter alia*, host cells comprising the adenoviral vector that has a heterologous region essential for encapsidation, what types of host cells are contemplated, additional components to this host cells that can also be included, pharmaceutical compositions comprising this adenoviral vector, diseases that can be treated, how to manufacture the pharmaceutical composition, how to administer the pharmaceutical composition, dosages to administer, acceptable formulations, and combining the adenoviral vector with other substances to improve the transfection efficiency. Despite this lengthy list of disclosure in column 12 of the '290 patent, it still does not appear to discuss BAV-3 vectors with a transgene inserted into the E3 region.

Applicant submits that if '290 patent is read in its entirety, it is evident that the subject matter of the patent and the teachings stemming from it have little relevance to the instant invention. The Lusky '290 patent is directed to adenovirus compositions and making adenoviruses with chimeric encapsidation regions whereby essential regions for encapsidation are swapped between human adenovirus 5 (hAd5, for short) and BAV3 (see col. 3 lines 17-25; col. 3 lines 52-56; and Example 2). The examples (columns 13-17) discuss the construction of a hAd5 (human adenovirus 5) vector with hAd5 E1 region and hAd5 E3 region deleted and two encapsidation regions inserted (one autologous encapsidation region from hAd5 and one heterologous encapsidation region from BAV3). The specification teaches that the adenoviral vector is preferably defective for at least the E1 region (col. 5, lines 63-65). Since the E1 region is an essential gene, the adenovirus vectors of the '290 patent are replication-defective. Example 2 describes the construction of a hAd5 vector with the hAd5 E1 region and hAd5 E3 region deleted with the insertion of the BAV3 encapsidation regions upstream of the native encapsidation region (col. 14, line 44-63). It is important to note that the encapsidation region of the adenovirus is located at the left hand end of the adenovirus genome (see col. 5, lines 12-16 of the '290 patent) while the E3 region (as in the instant claims) is located in the right hand end of the adenovirus genome. As such, the '290 reference neither describes nor suggests the claimed invention, which recites the limitations of being replication-competent. Furthermore, the '290 patent is silent in its teachings of whether any of the inserted genes are susceptible to splicing events, which is another limitation of the claimed invention.

As such, Applicant submits that '290 patent does not teach the construction of BAV-3 vectors, as the Examiner alleges at the top of page 4 of the Office Action. The '290 patent cannot serve as a basis of a combination of references for an obviousness rejection because the subject matter of the '290 patent is completely different from that of the current invention. The '290 patent is directed to making adenovirus vectors with different encapsidation regions. In contrast, the instant invention is directed to making bovine adenovirus vectors with an intron located 5' to a transgene that is susceptible to splicing inserted into the E3 region of BAV. None of these elements are mentioned nor suggested in the '290 patent. Accordingly, this reference cannot be combined with the other two references because there is no suggestion or motivation for one of skill in the art

to combine a reference teaching vectors with different encapsidation regions with Matthews or Kaufman.

Turning to the Matthew and Kaufman references, the Examiner alleges on page 3 of the Office Action that the intron of Kaufman is the same as used in Matthews and that the reference is provided to show features not explicitly taught in Matthews. However, the Examiner does not specify which features he is referring to in the Kaufman reference so it is difficult for the Applicant to rebut each particular feature when the Examiner does not specify to which particular features he is referring.

The Examiner also alleges that Matthews teaches "that the transgene is inserted in the E3 region (page 345, column 1 first [sic] paragraph)." Applicant respectfully disagrees with the Examiner's characterization. The description on page 345, left hand column, of "[u]sing vectors containing RG gene in E3" does not refer to the data in the Matthews paper but rather refers to the work done by Yarosh et al. (*Vaccine*, 14: 1257-1264 (1996). By making the quoted statement, authors of the Matthews reference were giving the background of the research that led to their work. A copy of the Yarosh reference is attached for the Examiner's convenience. In the Yarosh reference, the authors were working with human Ad5 virus (hAd5) and inserting RG into the E3 region of hAd5. Thus, the disclosure of Yarosh is directed to hAd5 E3 region and not BAV E3 region. This is significant because Zakhartchouk et al. (*Virology*, 250: 220-229 (1998)) discussed on page 220 that the E3 region of bovine adenoviruses is distinct from human Ad5 virus (hAd5) with its smaller size. More specifically, the distinctive features of bovine adenovirus E3 region is that there is an absence of additional leader (x, y, and z) sequences from fiber mRNA, and the absence of VA RNA genes from their usual locations. As such, the reliance upon Matthews is unfounded because the passage in Matthews that the Examiner was relying upon referred to another paper (Yarosh) and this Yarosh reference discusses hAd5 and not BAV.

Furthermore, the Matthews reference should not serve a reference for combination for an obviousness rejection because hAd5 was used and also because the construct in Matthews was inserted into the E1 region (an essential region). The instant invention is directed to *bovine*

adenoviruses and the insertion is into the E3 region (a non-essential region). As discussed above, the features of E3 in bovine adenoviruses are distinct from the features of human adenoviruses (including hAd5), thus it cannot be assumed that any teaching related to hAd5 would work the same in a bovine adenovirus system. Given that the Lusky '290 patent was directed to using different encapsidation regions in a human and bovine adenovirus system with no discussion or suggestion about inserting a heterologous transgene that is susceptible to splicing into the E3 region of BAV and that the Matthews reference focuses on fine-tuning the lac repressor system for human adenovirus vectors with insertions into the E1 region to make replication-defective vectors, one of skill in the art would not be motivated to combine two references with disparate subject matter.

The Examiner cites the use of the Kaufman reference to remedy the deficiencies of Matthews but the Examiner does not explicitly state what features discussed in Kaufman are being used in support of his arguments. The Kaufman reference does not even concern adenovirus vectors. Rather, the Kaufman reference reports the construction of plasmids to express the dihydrofolate reductase (DHFR) genes. The only item remotely related to adenoviruses in this reference is the use of the major late promoter (MLP) from human adenovirus 2. Applicant fails to see how a reference that does not even concern the construction of an adenovirus vector can be used in combination with the other references for an obviousness rejection for claims directed to bovine adenoviruses with an intron located 5' to a heterologous transgene that is susceptible to splicing and is inserted into the E3 region of the bovine adenovirus. The Kaufman reference does not discuss, teach, or suggest a bovine adenovirus vector with an intron located 5' of a heterologous transgene that is susceptible to splicing and inserted into the E3 region of bovine adenoviruses.

Applicants maintain the Examiner has not met his burden of showing a *prima facie* case of obviousness because one of skill in the art would not be motivated to combine the Lusky '290 patent (chimeric encapsidation regions), the Matthew reference (replication-deficient hAd5 vectors with insertions in the E1 region) and the Kaufman reference (plasmids to express DHFR using a human adenovirus 2 major late promoter).

2. There is No Reasonable Expectation of Success When these References are Combined.

Applicants submit that the Examiner has not met his burden of showing a *prima facie* case of obviousness because he has not shown a reasonable expectation of success when these three references are combined. As discussed above, the Lusky '290 patent is directed to a wholly different subject matter than the instant invention. The encapsidation regions discussed in column 5, lines 12-18 are located in a completely different region (i.e., the opposite end of the genome) than the E3 region that is recited in the instant claims.

The combination of Lusky with Matthews fails to produce any reasonable expectation of success since Matthews discusses human adenovirus 5 vectors with insertion into the E1 region to produce a replication-defective vector. As such, one of skill in art would recognize that there is no any reasonable expectation of success in combining these two references in attempts to produce a bovine adenovirus vectors with an intron located 5' to the transgene which is susceptible to splicing and is inserted into the E3 region. First, the adenovirus species are not the same. This fact is important because the transcriptional organization of E3 is different between the human adenoviruses and bovine adenoviruses (see the Zakhartchouk reference, discussed above). As such, there would be no expectation of success for one of skill in the art when trying to apply the teachings of the E3 region of hAd5 to the E3 region of BAV. Secondly, not only is the wrong species being discussed in the cited references but the region of the adenovirus into which heterologous DNA is being inserted is different as well. This results in completely different outcomes in terms of viral viability. The adenovirus vectors of Matthews results in replication-defective viruses whereas the instant claimed invention recites the necessary limitation of being "replication-competent." The Examiner asserts that the Matthews reference teaches replication competent vectors on page 345. However, as discussed above, this passage refers to the Yarosh paper used for background information and the Yarosh constructs are all *human* adenovirus vectors using promoters to drive expression. Thus, Matthews does not teach replication-competent *bovine* adenoviral vectors. Accordingly, one of skill in the art would realize that there is no reasonable expectation of success to obtain a replication-competent bovine adenovirus vectors with an intron

located 5' to the transgene which is susceptible to splicing and is inserted into the E3 region (the claimed invention) when one of skill in the art combines a reference for optimizing a lac repressor system for a replication-defective virus construct (Matthews) with a reference for making adenovirus vectors with chimeric encapsidation regions (Lusky). Third, the Applicant notes that the combination of both references still is silent on the heterologous transgene being susceptible to splicing. As such, there can be no reasonable expectation of success when the references do not teach nor suggest this feature which is a necessary limitation of the claimed invention.

As such, Applicant submits that no reasonable expectation of success has been shown and accordingly, no *prima facie* case of obviousness has been proved.

3. The Prior Art References Does Not Teach or Suggest All of the Claimed Limitations.

Applicant submits that the combined references do not teach or suggest all of the features of the invention as presently claimed. The combination of the Lusky '290 patent (directed to chimeric encapsidation regions), the Matthew reference (replication-deficient human adenovirus 5 vectors with insertions in the E1 region) and the Kaufman reference (plasmid to express DHFR using a human adenovirus 2 major late promoter) fails to teach of suggest all the elements listed above.

In view of the foregoing, Applicant submits that the Examiner has not proven any of the three requirements necessary to establish a case of *prima facie* obviousness. As such, Applicant respectfully requests that the Examiner withdraw the rejection and allow the pending claims.

## CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 293102002900. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Respectfully submitted,

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# Human adenovirus type 5 vectors expressing rabies glycoprotein

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*The prevalence of wildlife rabies throughout the world and the continued spread of this disease in North America highlights the need for oral vaccines which may be used safely and effectively to vaccinate a number of species that are reservoirs or vectors of rabies. We have previously shown that AdRG1, a replication competent recombinant human adenovirus type 5 (Ad5) expressing a rabies glycoprotein (RG), can induce immunity to rabies in rodent, canine, and skunk model systems. To improve the Ad5 vector system as a potential oral vaccine, we have constructed additional Ad5 recombinant vectors and compared RG expression in cell culture and immunogenicity in animals. Two new replication competent vectors are compared. AdRG1.3, which carries RG with accompanying SV40 polyA addition sequences within an E3 deletion, and AdRG4, which has RG in the E3 deletion but under the control of an exogenous Ad2 major late promoter, both express higher levels of RG in permissive cell culture than did AdRG1 and both elicit high levels of serum anti-rabies antibodies by parenteral or oral routes in animals. AdRG1.3 may be a more effective vaccine vector in species which are non-permissive for the replication of human Ad5. Copyright © 1996 Elsevier Science Ltd.*

**Keywords:** Adenovirus vector; rabies vaccine; expression vector

Vaccination against rabies continues to be the only effective means to prevent disease following rabies infection. An immunization program of at-risk wildlife species reduces the number of non-protected animals so that the virus is not sustained in nature[1,2] and therefore minimizes the probability of virus infection of domestic animals or humans. Significant reservoirs of rabies in fox, and jackal populations and occurrences of the virus in bats and in cattle contribute to the risk of infection[3,4]. In North America, the continuing spread of a raccoon rabies epizootic in the mid-Atlantic and northeastern United States and a recent outbreak of canine rabies in coyotes in Texas has increased the risk of contact between afflicted animals and humans in rural as well as in heavily-populated urban centres[5–7]. Attenuated rabies vaccines which are effective in baiting programs designed to immunize canid species[2,8] often fail to effectively immunize other host species such as skunks and raccoons and these vaccines may be pathogenic in rodents[2,9–11]. Recombinant viruses expressing rabies glycoproteins (RGs) as vaccines have considerable potential for overcoming some of these problems. RG can induce protective virus neutralizing antibodies[12–15] and MHC class I- or class II-restricted cytotoxic T cells[16–18] and vaccinia virus vectors expressing this antigen are currently being evaluated as oral vaccines[19–21]. Adenovirus-vectored vaccines will be a useful adjuct vaccine with vaccinia virus vectored vaccines[22] and may be a suitable alternative vaccine in some situations[23].

Human adenovirus type 5 (Ad5), used as a viral vector to express foreign antigens has many desirable properties in applications as a recombinant vaccine[24,25]. We have previously described AdRG1, a replication-competent Ad5 vector in which E3 sequences (79.6–84.8 map units) have been replaced with a cassette consisting of the SV40 early promoter, rabies (ERA strain) glycoprotein gene and the SV40 polyA sequences[26]. In several species, such as mice, dogs or skunks, which are at best only semi-permissive for Ad5 replication, good titres of protective anti-rabies antibody were induced after oral, intranasal or parenteral vaccination with AdRG1[26,27]. However, to obtain a good immune response in a large proportion of treated animals, particularly for oral vaccination, high doses of this vector were required. Since it seemed likely that the immune response would correlate with the level of RG produced by the vector, we investigated ways of increasing gene expression.

We describe the construction of recombinant Ad5 vectors with the RG gene placed within a deletion in the E3 transcription unit and under the control of endogenous or exogenous adenovirus-based promoters. The synthesis of RG by these vectors was assessed in cell culture and the immunogenicity of the vectors determined by intraperitoneal (i.p.) injection in mice and

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direct oral administration to striped skunks (*Mephitis mephitis*).

## MATERIALS AND METHODS

### Cells and viruses

The 293 cell line[28] was maintained in MEM F-11 supplemented with 10% (v/v) newborn calf serum and antibiotics (Gibco BRL Life Technologies Inc., Burlington, ON) as previously described[29]. HeLa and Madin–Darby canine kidney (MDCK) cells were grown in $\alpha$-MEM supplemented with 10% (v/v) newborn calf serum and antibiotics.

Wild type human Ad5 (Ad5), AdRG1[26] and other recombinant Ad5 vectors were grown in 293 or HeLa cells and titred on 293 cells as described by Graham and Prevec[25,30]. Adenovirus vectors developed in this work are described in the text.

### Construction of plasmids

The following plasmids were used during vector construction: pSV2X3RG containing the ERA strain RG gene between the SV40 early promoter and polyadenylation sequences[26], pFG144K3[31], pAB14[32], pFG173[32] a derivative of pFG140 containing all of the Ad5 genome except for a lethal deletion near the E3 region, pKDAdMLP-5a containing the Ad2 major late promoter (MLP), a fusion of the tripartitite leader sequence and 5′ and 3′ splice sites[33] (obtained from J.A. Hassell, McMaster University, Hamilton, ON). Plasmids constructed as part of this study are described in subsequent sections. Restriction endonucleases and DNA modifying enzymes were acquired from Gibco BRL Life Technologies, Inc., New England BioLabs Ltd (Beverly, MA), Boehringer-Mannheim Canada, Inc. (Laval, QB) and Pharmacia Canada, Inc. (Baie d'Urfé, QB). Oligonucleotides were acquired from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, ON).

### Generation of recombinant viruses

Two new recombinant Ad5 viruses were constructed as described below. One (AdRG1.3) contains the RG cDNA sequence substituting for Ad5 E3 so as to allow expression from endogenous Ad5 promoters. In contrast, the other (AdRG4), has the RG sequence in Ad5 E3 downstream of an exogenous Ad2 MLP element and the resultant expression cassette is placed within E3 in a antiparallel orientation so that neither the endogenous E3 promoter nor Ad5 MLP can be used to transcribe RG in this construct.

For the construction of AdRG1.3, an oligonucleotide linker sequence (5′ CATGTCTAGA 3′), was fist ligated into the *Nco*I site of plasmid pSV2RG1[26]. This introduced a *Xba*I site immediately upstream of the RG translation initiation site into the resultant plasmid pSV2RG1a and at the same time alters the sequence around the translation initiating ATG from TCC ATG G to GAC ATG G. This change might be expected to improve translation efficiency since a G residue, rather than a T, three bases upstream of the ATG, more closely fulfills the requirement for efficient translation as defined by Kozak[38]. The purified 1.8 kb *Xba*I fragment from this plasmid, corresponding to RG and SV40 polyA sequences, was ligated into the *Xba*I linker site in pAB14. The resulting plasmid pOYRG1.3, which carries the introduced sequences within an E3 region *Bgl*II deletion (nt 28,133 to 30,818) in parallel to the E3 promoter, was cotransfected into 293 cells with pFG173 to obtain AdRG1.3.

AdRG4 was designed to express RG as a result of transcription driven by an exogenous Ad2 MLP. The Ad2 MLP, tripartite leaders cDNA, and 5′ and 3′ splice sites was obtained as a 0.75 kb *Sac*II/*Bam*HI fragment from pKBAdMLP5a and after treatment with Klenow polymerase to produce blunt ends, was ligated with the large *Nco*I fragment of pSV2RG1, also with blunt ends produced by Klenow polymerase. As determined by sequencing, a fortuitous aberrant ligation between the Klenow enzyme-treated *Bam*HI and *Nco*I sites produced a translation initiation sequence (GAC*ATG*G) for the RG in resultant plasmid pRG/MLP, which was identical to that in the RG1.3 construct described above. The *Xba*I fragment from pRG/MLP, containing a substantial portion of the SV40 early promoter-enhancer region, Ad2 MLP, RG and SV40 polyA sequences, was inserted into the *Xba*I site (nt 28592–30470) of pFG144K3 in antiparallel orientation relative to the E3 promoter. The resultant plasmid (pPBRG4), was cotransfected into 293 cells with pFG173 to generate AdRG4.

Plasmid DNA cotransfections to rescue cloned viral sequences into full length viral genomes were carried out by the calcium phosphate protocol as previously described[30,34]. Potential recombinant virus isolates were plaque purified at least twice on 293 cells, and viral DNA was prepared and analysed by restriction digestion with the appropriate enzymes. Viral stocks were prepared as described by Graham and Prevec[30].

### Antibodies

Mouse monoclonal antibody (MAb) 10ED8·2B8 against RG (A. Wandeler, unpublished) as well as MAb H2-19 specific for Ad5 72K protein[35], and MAb 9F6-6 specific for Ad5 hexon protein (J.F. Williams, Carnegie Mellon University, Pittsburgh, PA) were used in this study.

### Viral protein expression

Cells, at 80–90% confluency, were washed with $PBS^{2+}$ [phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$) with 0.68 mM $CaCl_2 \cdot 2H_2O$ and 0.49 mM $MgCl_2 \cdot 6H_2O$] and virus was adsorbed for 1 h at 37°C. To examine protein expression in the absence of DNA synthesis, a 1:20 dilution of 1-$\beta$-D-arabinosyl cytosine (araC) (2 mg $ml^{-1}$ in sterile $dH_2O$) was added per dish at the time of infection, at 1 h post-infection (p.i.), and every subsequent 7 h. Prior to radiolabelling, serum supplemented medium 199 lacking methionine (199-met) replaced the normal growth medium for 30 min. For radiolabelling, 199-met containing 50 $\mu$Ci Trans [$^{35}$S] label (ICN Radiochemicals, Inc., St. Laurent, QB) was added per dish for 2 h. Cells were harvested into prechilled RIPA buffer [0.05 M Tris·HCl (pH 7.2), 0.15 M NaCl, 0.1% (w/v) sodium dodecyl sulfate, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100], and extract passed through a 22G needle to shear the DNA.

Radiolabelled glycoprotein was immunoprecipitated with anti-rabies antibodies and analysed on polyacrylamide gels as previously described[30].

### Immunization of mice and skunks

Female BALB/c mice, 6–8 weeks old (Charles River Laboratories, QB), were immunized by i.p. injection of virus in 100 $\mu$l of PBS, and subsequently housed in a Level II isolator unit with food and water *ad libitum*. Mice were bled under anaesthesia by retro-orbital puncture 1 month after immunization.

Striped skunks (*Mephitis mephitis*) were acquired from Ruby's Fur Farm, New Sharon, IA. Two millilitres of each vaccine dilution was given to eight skunks by direct instillation into the oral cavity. A syringe fitted with a 5 cm long polyethylene tube was used to deposit vaccine directly into the mouth of the skunks. The animals were restrained by hand for this procedure. Blood samples were taken from jugular veins before and at various times after vaccination from animals anaesthetized with a 5:1 mixture of ketamine-hydrochloride and acepromazine maleate.

### Rabies virus antibody assays

Antibody titres in mouse sera were determined by an indirect ELISA method, with purified rabies (ERA strain) viral glycoprotein as antigen[36]. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was used for detecting mouse antibodies. Although the method measured antibody binding rather than virus neutralization, previous studies[36] have shown that there is generally good correlation of this assay with results of the fluorescent inhibition microtest[37] (FIMT) which measures virus neutralizing antibodies[36]. The FIMT assay was used for measuring the anti-rabies immune response in skunk sera. Our previous studies with the same rabies antigen, expressed by AdRG1, have shown that all mice[26] or skunks[27] showing an immunological response to the RG following immunization with the AdRG1 recombinant, were protected against a subsequent challenge. Animals given an adenovirus not expressing RG did not develop a measurable anti-rabies immune response by these assays. Based on this experience no challenges of immunized animals were carried out.

Responses of the animals to vaccine injection are presented as the median titre. Since eight skunks were used per vaccine dose in many cases two median values are given since an equal number of animals responded at titres above and below the presented values.

## RESULTS

### Kinetics of expression of RG in permissive and non-permissive cell culture

These experiments compare RG expression in cell culture following infection with each of three replication competent Ad5 vectors all carrying the RG gene within a deletion of the Ad5 vector E3 coding region. AdRG1, which was previously described[26], has the RG gene and accompanying SV40 immediate early promoter and polyA addition sequence in the E3 parallel orientation. AdRG1.3, is identical to AdRG1 except that the SV40 promoter is deleted and the sequence around the translation initiating ATG of the RG gene has been modified as described in Materials and Methods. AdRG4 has the RG gene accompanied with an Ad2 MLP with spliced leader sequences and an intron sequence all inserted in the E3 antiparallel orientation. *Figure 1* is a pictorial representation of these recombinant vectors.

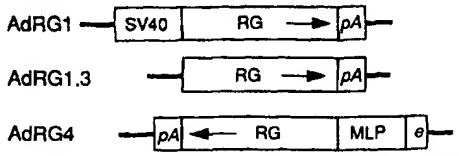


**Figure 1** Schematic of RG and accompanying exogenous elements, inserted into deletions within the E3 region of human Ad5. The details of AdRG1 construction have been previously described[26]. In AdRG1 the RG gene is accompanied by SV40 immediate early promoter and polyA addition signal while in AdRG1.3 the SV40 promoter has been deleted. Both AdRG1 and AdRG1.3 have the RG sequences in parallel to the transcription direction of Ad5 E3 and MLP transcripts. In AdRG4 the RG gene is accompanied on the upstream side by a modified Ad2MLP containing three spliced leaders and an intron sequence as well as upstream promoter-enhancer elements (e) from the SV40 promoter, and on the downstream side by the SV40 polyA addition signal. AdRG4 is oriented so that the RG gene is antiparallel to the host Ad5 E3 and MLP transcripts. The exogenous sequences in AdRG1 and AdRG4 are placed within an *Xba*I deletion (nts 28592–30470) of Ad5 E3 while AdRG1.3 contains its insert between two *Bgl*I sites in the Ad5 E3 region (nts 28133–30818)

The kinetics of expression of RG by AdRG1, AdRG4 and AdRG1.3 was examined using 2 h labelling pulses at 6 h intervals over a 24 h period in infections of HeLa and MDCK cells (*Figure 2*). The activity of the endogenous MLP in RG expression was estimated by comparing RG synthesis in the absence and presence of the DNA synthesis inhibitor araC. To confirm that late protein synthesis was blocked by the DNA synthesis inhibitor, no synthesis of Ad5 hexon protein was observed in araC-treated cultures (data not shown). As a control for the efficiency of Ad5 infection, the synthesis of Ad5 72K E2 gene product was also determined by immunoprecipitation as seen in *Figure 2*.

A comparison of RG expression by the three vectors in permissive HeLa cells showed that the maximal rate of synthesis occurred at *ca* 12 h for all three vectors (*Figure 2a*). As expected from previous results[26], only low levels of the RG product made by the AdRG1 vector was detected under these conditions of labelling and film exposure. In contrast, significantly greater amounts of RG were detected in HeLa cells infected with the new vectors AdRG1.3 and AdRG4. Synthesis of the RG protein by AdRG1 or AdRG1.3 was unaffected by araC while expression from AdRG4 was decreased some 50% in the presence of the drug. As will be considered more fully in the Discussion these results suggest that expression of RG in AdRG1 and AdRG1.3 is driven mainly by promoters which are independent of viral DNA synthesis while the Ad2 MLP functions to some extent even in the presence of a DNA$^9$ synthesis inhibitor[39,40,41].

In non-permissive MDCK cells no expression of RG by vector AdRG1 was detectable under the conditions used (*Figure 2b*). This is in agreement with the generally low level of expression by this vector. In contrast, vector AdRG1.3 expressed readily detectable levels of RG in MDCK cells, a not unexpected finding since an Ad5 vector containing a lacZ gene in a similar context as the RG gene in AdRG1.3, produces high levels of

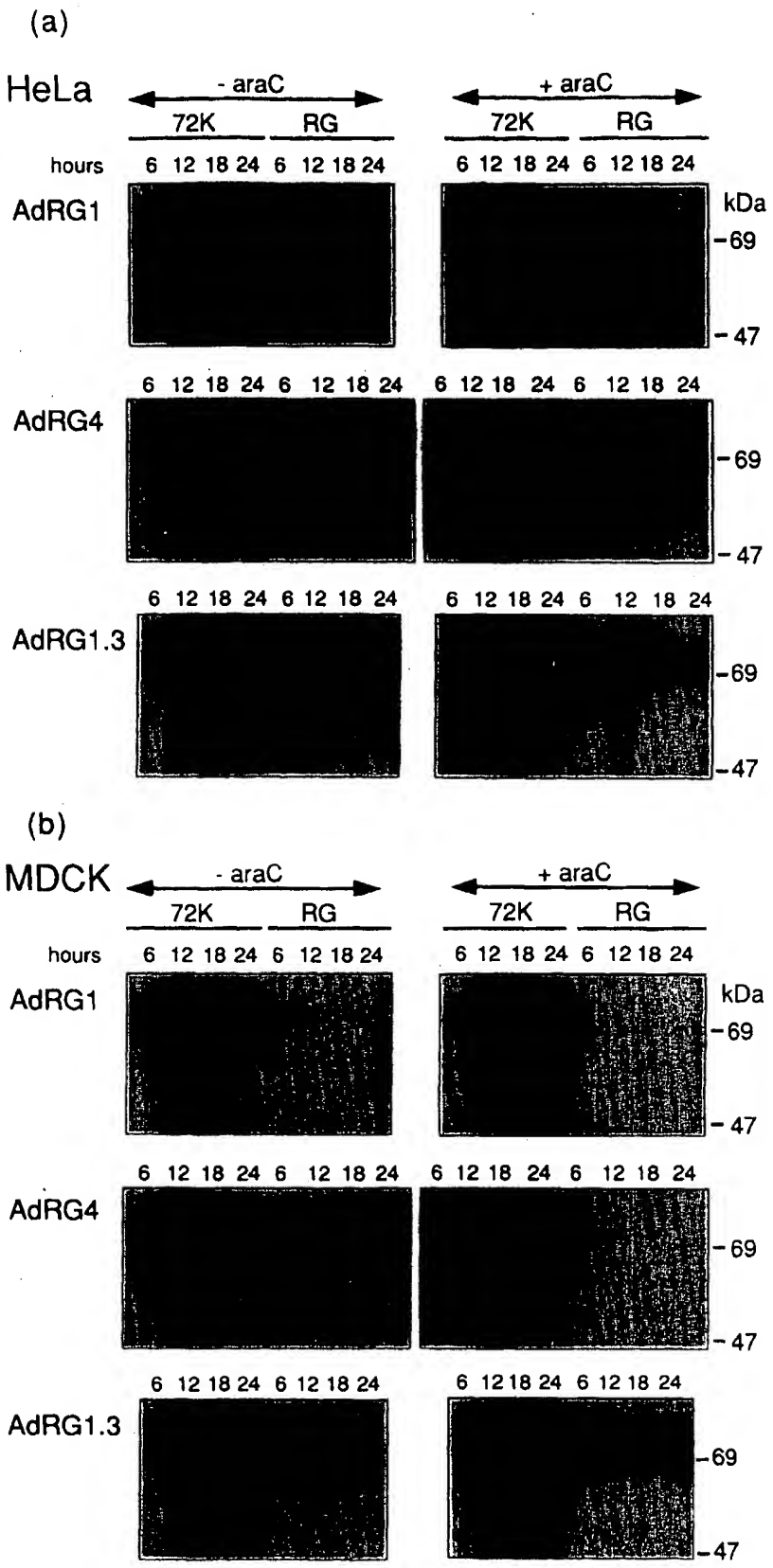


**Figure 2** Kinetics of synthesis of RG in HeLa and MDCK cells following infection with vectors AdRG1, AdRG4 and AdRG1.3 in the presence or absence of araC. Cells were infected with the indicated virus and radiolabelled with Tran [$^{35}$S] label for 2 h ending at the indicated time. The infected cell proteins were immunoprecipitated with antisera to either Ad5 72K protein or to rabies glycoprotein. (a) Results from permissive HeLa cells infected with each of the vectors and labelled at 6, 12, 18 and 24 h either in the continuous presence of araC or in its absence. (b) A parallel experiment carried out in non-permissive MDCK cells. The radioactivity seen in the 6 h time point following immunoprecipitation with RG is almost exclusively due to spillover from the adjacent 72K immunoprecipitate lane. The numbers 69 kDa and 47 kDa mark the position in the gel of markers with this molecular weight

**Table 1** Serum immune response of mice immunized i.p. with Ad5 vectors expressing RG

| Ad5 Vector | | | |
|---|---|---|---|
| Titre | Virus | Responders/total | Antibody titre Median[a]+range |
| $10^8$ | AdRG1 | 5/5 | 256 (256–512) |
| | AdRG4 | 5/5 | 512 (128–512) |
| | AdRG1.3 | 5/5 | 256 (256–512) |
| $10^7$ | AdRG1 | 5/5 | 256 (256–512) |
| | AdRG4 | 4/5 | 512 (<8–512) |
| | AdRG1.3 | 5/5 | 256 (16–256) |
| $10^6$ | AdRG1 | 5/5 | 128 (16–256) |
| | AdRG4 | 5/5 | 128 (16–256) |
| | AdRG1.3 | 5/5 | 256 (128–256) |
| $10^5$ | AdRG1 | 4/5 | 16 (<8–32) |
| | AdRG4 | 4/5 | 64 (<8–128) |
| | AdRG1.3 | 3/5 | 64 (<8–128) |

[a]Median includes non-responders and indicates titre of mid-point animal

β-galactosidase in MDCK cells[42]. What was somewhat surprising was the apparent lack of significant RG synthesis by the AdRG4 vector in MDCK cells. Since, as shown above, this vector can express RG in HeLa cells with the kinetics of an early viral gene, it might be expected that expression would occur in the MDCK system. As will be discussed later it is possible that the rate limiting step in this situation may be at the post-transcriptional level.

### Serum antibody response to rabies glycoprotein in inoculated mice

BALB/c mice were immunized i.p. with recombinant vectors AdRG1, AdRG1.3, and AdRG4 to compare the anti-rabies responses as measured by the production of rabies antibodies reacting in an ELISA assay. Seroconversion was observed in all mice given high doses of any of the recombinant viruses ($1 \times 10^8$ p.f.u.), and in all except one given $10^7$ p.f.u. (*Table 1*). A dilution series of inoculations of the three recombinants confirmed the dose response seen previously with AdRG1[26]. Although animals in the present study were not challenged with infectious rabies virus, we have shown previously[26] that all vaccine-treated mice with any measurable serum antibody titre will survive intracerebral challenge with normally lethal doses. At the lowest immunizing doses of vector ($10^5$ p.f.u.), the level of antiserum induced in responding animals by AdRG4 or AdRG1.3 appears to be greater than that induced by AdRG1 but the differences between the vectors in this semi-permissive host species are minimal.

### Serum antibody response in skunks given recombinant vectors by the oral route

To complement the study in mice and to determine the practicality of using these vectors as oral vaccines, AdRG1, AdRG1.3 and AdRG4 were compared for their ability to induce an immune response in striped skunks (*M. mephitis*) following instillation of a 2 ml dose of virus directly into the oral cavity (DIOC). Post-immunization serum was tested for the production of rabies neutralizing antibody by FIMT (*Table 2*). Positive responses were detected in some animals at 14 days after immunization and maximum neutralizing titres were detected at 30–60 days post-immunization, although several animals failed to develop an immune response until day 90. At the lowest vaccine dose ($1–2 \times 10^5$), non-responding animals were frequently observed (*Table 2*). A significant correlation between the initial dose of vaccine and the neutralizing titre was observed. More importantly, the duration of immunity observed with all vectors, when used at sufficiently high immunizing dose, supports the suitability of vectors of this type for a wildlife baiting program.

From the data presented in *Table 2*, it is clear that AdRG1.3 is both more effective at lower doses (i.e. induces a response in a larger proportion of animals) and induces higher levels of antibody in responding animals when compared to the other two vectors.

## DISCUSSION

The experiments described in this paper demonstrate the improved immunogenicity of infectious Ad5 vectors that express the RG at levels higher than those previously observed with the original vector AdRG1[26]. As described in results, the vector AdRG1.3 differs from AdRG1 by the deletion of an exogenous SV40 promoter and the alteration of the sequence around the translation initiating ATG from TCC ATG G to GAC ATG G. The effect of this latter change was not directly investigated but was expected to result in improved translation efficiency[38]. AdRG1.3 produced high levels of RG expression at both early (6 h) and late times (18 h) in infections of permissive human cells as well as in non-permissive MDCK cells. In the presence of araC levels of glycoprotein expression were unaffected suggesting that RG transcription in AdRG1.3 occurs primarily from the Ad5 E3 promoter throughout the virus replication cycle with little or no contribution by the endogenous MLP.

As has previously been described, exogenous promoters inserted into E3 in an orientation parallel with the E3 transcription direction, often have little direct effect in promoting expression of the downstream gene. Constructs expressing HSV-1 gB from a vector containing the SV40 promoter[43], or a vector with the VSV glycoprotein and the HSV-1 thymidine kinase promoter[44] were shown to have mRNA transcripts originating upstream of the exogenous promoter. Mittal *et al.*[45] showed that expression of β-galactosidase from a lacZ gene was independent of DNA synthesis and was highest when no SV40 promoter was present. In contrast, this same paper shows that expression of luciferase from similar Ad5 constructs occurred only after DNA replication and was greatly augmented by the presence of an SV40 promoter[45]. Expression only in presence of viral DNA synthesis suggests that the SV40 early promoter functions not as a promoter for the luciferase gene but may function as a potential splice acceptor site for transcripts arising upstream, probably at the endogenous MLP, as described for the HSV-1 gB vector[43]. The general conclusion that might be drawn from these results, and which would be consistent with our observations with the rabies constructs described in this paper, is that genes expressed to high levels from the endogenous E3 promoter may be inhibited by the presence of an upstream exogenous promoter. In contrast,

**Table 2** Immunization of skunks with rabies glycoprotein vectors

| Virus | Dosage[a] | Responders Total | Range antibody titres (median values in each group)[b] | | | | | |
|---|---|---|---|---|---|---|---|---|
| Study 1 | | | Day 0 | Day 14 | Day 30 | Day 60 | Day 92 | Day 126 |
| AdRG1 | $2.0\times10^7$ | 7/8 | <8 (<8) | <8–256 (<8) | <8–512 (8,32) | <8–2048 (32) | <8–512 (16) | <8–512 (16,32) |
| AdRG1 | $2.0\times10^6$ | 7/8 | <8 (<8) | <8–64 (<8) | <8–2048 (<8) | <8–2048 (16,32) | <8–2048 (32,64) | <8–2048 (16,128) |
| AdRG1 | $2.0\times10^5$ | 2/8 | <8 (<8) | <8 (<8) | <8 (<8) | <8 (<8) | <8–64 (<8) | <8 (<8) |
| AdRG4 | $2.0\times10^7$ | 7/8 | <8 (<8) | <8–256 (16,64) | <8–4096 (512) | <8–4096 (1024,2048) | <8–4096 (256,512) | <8–4096 (512) |
| AdRG4 | $2.0\times10^6$ | 8/8 | <8 (<8) | <8–16 (<8,8) | 8–512 (16,32) | 32–256 (32,64) | 16–512 (32) | 8–512 (32) |
| AdRG4 | $2.0\times10^5$ | 2/8 | <8 (<8) | <8 (<8) | <8–64 (<8) | <8–16 (<8) | <8–32 (<8) | <8 (<8) |
| Study 2 | | | Day 0 | Day 14 | Day 30 | Day 52 | Day 91 | |
| AdRG1 | $2.0\times10^7$ | 7/8 | <8 (<8) | <8–4096 (32,128) | <8–8192 (1024) | <8–4096 (512,2048) | <8–4096 (256,512) | |
| AdRG1 | $2.0\times10^6$ | 7/8 | <8 (<8) | <8–256 (<8) | <8–4096 (128,256) | 8–2048 (256,512) | <8–1024 (128) | |
| AdRG1 | $2.0\times10^5$ | 3/8 | <8 (<8) | <8 (<8) | <8–256 (<8) | <8–512 (<8) | <8–256 (<8,8) | |
| AdRG1.3 | $2.0\times10^7$ | 7/8 | <8 (<8) | <8–4096 (512,2048) | <8–8192 (8192) | <8–16384 (4096) | 16–8192 (1024,2048) | |
| AdRG1.3 | $2.0\times10^6$ | 8/8 | <8 (<8) | 8–2048 (128) | 64–4096 (256,512) | 128–8192 (512,2048) | 32–2048 (256,2048) | |
| AdRG1.3 | $2.0\times10^5$ | 7/8 | <8 (<8) | <8 (<8) | <8–128 (32,64) | <8–256 (64,128) | <8–1024 (64) | |

[a]Skunks were administered a 2 ml dose of vaccine directly into the oral cavity; [b]medians include non-responders and indicate an equal number of animals above and below the indicated titres

genes expressed primarily from the endogenous MLP, i.e. inhibited by DNA synthesis inhibitors, may utilize sequences in the exogenous promoter as cryptic splice acceptor sites to allow inclusion of the inserted gene sequence into mRNA. A corollary to this conclusion would be that some inserted genes, depending on their sequences, may be inappropriately spliced or unable to complete splicing when transcribed from one of the two endogenous viral promoters.

In contrast to AdRG1.3, which is driven by endogenous Ad5 promoters, the recombinant vector AdRG4 has the RG gene under the control of the Ad2 MLP promoter and associated SV40 enhancer. The exogenous Ad2 MLP-driven cassette was placed within a deletion of Ad5 E3 genes in an orientation opposite to normal E3 transcription thus ensuring that expression due to transcripts from the E3 or endogenous MLP is impossible. Permissive HeLa cells infected with AdRG4 produced significant amounts of RG protein both in the presence and absence of araC. The ability of this vector to express RG in the absence of DNA synthesis, may be related to the close proximity of the RG gene to the tripartitite leader sequence, a situation analogous to transcription and expression of the L1 family which can also occur prior to DNA replication during normal adenovirus infection[39,40,41]. It is also possible that expression from the MLP in the absence of DNA synthesis is partly a function of the SV40 enhancer elements. In contrast to the result in HeLa cells, RG was not expressed to any significant level by AdRG4 in non-permissive MDCK cells. Thus despite the apparent ability of the Ad2 MLP element to function as an early promoter in HeLa cells, the gene product was not produced in the canine cell line, a situation in which we might have expected early promoters to continue to function as seen by the synthesis of the 72k protein in this experiment and from the work of Martins[42]. It is probable that the lack of RG expression in this instance is due, not to the lack of promoter activity, but rather to an inability of the RNA transcripts to undergo proper processing or to be efficiently translated in non-permissive host cells. Effects of this type have been observed for human Ad in non-permissive monkey cells[46].

The efficacy of recombinant adenovirus vectors in inducing neutralizing antibody responses against RG and providing protection against disease development was previously documented[26,27]. Hypothetically, lower doses of AdRG1.3 and AdRG4, vectors expressing moderate to high levels of RG in cell culture, might be adequate for a humoral response in mice. In a dilution series of immunizations (*Table 1*) we observed that a dose of $10^5$ p.f.u. given to mice produced a four- to fivefold higher mean antibody titre with AdRG1.3 and AdRG4 than with AdRG1, though differences between the vectors were less apparent at higher virus doses. Mouse cells, in general, are semi-permissive for Ad5 expression although virus replication is substantially slower in this species than in fully permissive cells.

Data obtained in this study have also provided information concerning the relative effectiveness of the Ad5-RG vectors in eliciting a humoral response following infusion into the oral cavity of skunks, a species not protected by the currently licensed vaccine[47]. Though AdRG1.3 was clearly more effective in both the levels of antibody induced at a specific dose and in the number of responding animals at lower doses, it was of interest that very little difference was observed between AdRG1

and AdRG4. This probably reflects the fact that neither of these viruses is a highly efficient producer of RG in non-permissive cells. This observation would be consistent with skunk epithelial cells being relatively non-permissive for Ad5 replication.

It has been observed that the doses of recombinant Ad-rabies vectors to be packaged into a blister pack bait need to be considerably higher than the minimum dose used in direct vaccine administration into the oral cavity of target species (A.I. Wandeler, unpublished observations). This may be due to inefficient delivery of the adenovirus vaccines to the appropriate tissue by currently used baiting systems. It is important therefore that, to a first approximation, our results indicate a correlation between levels of antigen expressed in cell culture and amounts of virus required to induce particular levels of antibody in animals.

Our observations of RG expression by recombinant adenovirus vector AdRG1.3 in non-permissive cell culture systems confirm the feasibility of vaccinating generally non-permissive wildlife host species with human adenovirus-based recombinant vaccines[26,27,44].

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